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INTERACTION OF GLUCOCORTICOID ANALOGUES WITH THE HUMAN GLUCOCORTICOID RECEPTOR

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Summary—Transient co-transfection of receptor cDNA and suitable reporter genes was used to study human glucocorticoid receptor (hGR) function in a neutral mammalian cell background. A variety of natural and synthetic steroids were analyzed for their ability to activate gene expression through the hGR and to bind to extracts of cells expressing the hGR cDNA. There was very good correlation between these two *in vitro* parameters for these compounds. Furthermore, correlation of these data with reported *in vivo* anti-inflamniatory potencies was surprisingly close, with two exceptions. The *in vitro* data suggest an explanation for the discrepant compounds, consistent with published data on their metabolic fate *in vivo*. The co-transfection assay has utility as a quantitative predictor of *in vivo* glucocorticoid pharmacology.

INTRODUCTION

The human glucocorticoid receptor (hGR), cloned in 1985 [1], is known to be a member of a protein superfamily of closely related intracellular receptors (IRs) which function as ligand-activated transcription factors [2–4]. The hormone–IR complex can positively or negatively regulate the expression of gene networks by its interaction with specific target hormone response elements (HREs) within the promoters of controlled genes. One useful development in the elucidation of the structure and function of the GR protein was the development of a "*cis–trans*" or co-transfection assay in which glucocorticoid-dependent transcriptional control could be reconstituted in a model cell system [5]. This has enabled significant advances in the understanding of the domain structure of the hGR and other IRs [5–7]. In the "*cis–trans*" assay, a plasmid encoding the cDNA for the hGR under a constitutive promoter, e.g. the Rous sarcoma virus (RSV) long terminal repeat (LTR), and a second plasmid carrying a gene for a detectable reporter, e.g. firefly luciferase (LUC), under the control of a glucocorticoid-responsive promoter, e.g. the LTR of the mouse mammary tumor virus (MMTV), are introduced into a neutral mammalian cell background. This results in reconstitution of hormone-dependent transcriptional transactivation of reporter gene expression. This introduction of the hGR cDNA and the MMTV LTR-LUC can be accomplished by preparing suitable adenoviral vectors [9] or by transient transfection of two plasmids, one directing overexpression of hGR and the other encoding MMTV LTR-LUC. In addition to conferring on recipient CV-1 cells a functional and measurable response to glucocorticoids, adenoviral infection or transient transfection with the hGR-encoding vector also confers specific binding of tritiated dexamethasone.

The availability of a reliable and quantitative *in vitro* predictor of *in vivo* anti-inflammatory activity would have significant benefit. In the present study, we evaluate the utility of *in vitro* quantitative assessment of various glucocorticoid analogues using the "*cis–trans*" assay and radioligand binding as predictors of *in vivo* activity of these compounds. The potency and efficacy of a panel of 21 natural and synthetic glucocorticoids were assessed. The functional agonist activity of these 21 glucocorticoids was determined in CV-1 cells expressing transfected hGR cDNA over a full range of concentrations (10^{-11} to 10^{-5} M). This was compared to their potency to displace specifically bound tritiated dexamethasone from cytoplasmic extracts of CV-1 cells expressing the hGR protein after introduction of hGR cDNA.

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EXPERIMENTAL

Media and chemicals

CV-1 cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (Gibco, Grand Island, NY) and supplemented with 2 mM L-glutamine (Gibco), and 55 μg/ml gentamicin (Flow Laboratories, McLean, VA). Plasmids pRShGR, pGRE-LUC, and pRSV-β-gal have been previously described [5]. Briefly, pRShGR is a pBR322 derivative containing the hGR cDNA under control of the RSV LTR. pGRE–LUC contains a cDNA for LUC under the control of the MMTV LTR, a conditional promoter containing a glucocorticoid response element (GRE). pRSV-β-gal contains the gene for *E. coli* β-galactosidase under control of the RSV-LTR, a constitutive promoter. Unlabeled chemicals were obtained from Sigma Chemical Co. (St Louis, MO). [^{3}H]Dexamethasone (approx. 40 Ci/mmol) was purchased from Amersham Radiochemicals (Arlington Heights, IL).

Buffers and enzyme assays

For the competitive binding assay, homogenization buffer [(10 mM Tris–HCl (pH 7.4) 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 2 mM dithiothreitol, 0.25 M sucrose)] and gelatin phosphate buffer [0.15 M NaCl, 0.015 M NaN_3, 0.1 M Na_2HPO_4, 0.039 M NaH_2PO_4 (pH 7.0), 0.1% gelatin] were utilized.

Buffers utilized for the calcium phosphate mediated co-transfection assay were as described [8]. All cell washing steps were in 150 mM phosphate buffered saline (PBS). Test ligands were prepared in DMEM containing 10% (v/v) charcoal-absorbed fetal calf serum. Luciferase activity in cell extracts was assayed in 9.7 mM $MgCl_2$, 1.66 mM ATP, 0.45 sodium luciferin, 91 mM potassium phosphate, pH 7.8 on a Dynatech luminometer as described [9]. Cell extracts were also analyzed for β-galactosidase activity as described previously [5].

Preparation of cell extracts

CV-1 cells were infected with Ad/MLUC7 [9], a recombinant adenovirus expressing the hGR cDNA, in the presence of dl309 helper virus (5 PFU/cell) by incubating cells and viruses in DMEM with 10% FBS (DMEM/FBS) at 37°C. After a 30 min adsorption, the mixture was diluted 10-fold with DMEM/FBS and plated in 15 cm culture dishes. At the end of an 18 h incubation at 37°C, cells were detached an washed with PBS by centrifugation at 700 g fo 5 min. All further procedures were carried ou at 4°C. Cells were homogenized in 2 vol o homogenization buffer in a Teflon homogeniz with a motor-driven pestle at 1250 rpm. Th homogenate was clarified by centrifugation a 1000 g for 15 min. A soluble cytosol fractio was generated by centrifuging this supernatar fraction at 104,000 g for 1 h. The protein cor centration of the cytosol fraction was deter mined by dye binding [10], using bovin γ-globulin as standard. Cytosol fractions wer used immediately or stored at −70°C.

Competitive binding assay

Aliquots of cytosol (100 μg protein) wer incubated at 4°C with 2.5 nM tritiated dexa methasone in the presence of incremental con centrations (0–2.5 × 10^{-5} M) of unlabele dexamethasone or unlabeled cold competito test compounds. After a 24 h incubation perio at 4°C, unbound steroid was removed by ad dition of 2 vol of 7.5% dextran-coated charcoa in gelatin-phosphate buffer. The mixture wa incubated for 10 min at 4°C and centrifuged a 3000 g for 10 min. The radioactivity in the supernatant fluid was determined by liquid scintillation counting. The non-specific binding wa determined in the presence of unlabeled dexamethasone (10^{-5} M). All determinations were performed in duplicate.

Co-transfection assay

Co-transfections were performed essentially as described [5]. Sub-confluent CV-1 cells were passed at 3 day intervals to maintain good transfection efficiency. CV-1 cells were plated 24 h prior to transfection at 70% confluency. The recombinant DNA constructs were transiently transfected into CV-1 cells by calcium-phosphate co-precipitation [8]. Each plasmid preparation used for transfection was cesium banded twice prior to use. Following transfection, all subsequent steps were performed on the Biomek Beckman Automated Workstation. Medium was removed from transfected cells after 6 h, cells were washed and each glucocorticoid was tested at seven incremental concentrations in duplicate (10^{-11}–10^{-5} M). After 38 h the cells were washed and lysed with 0.5% Triton-X 100 and assayed for luciferase and β-galactosidase activities, using a luminometer (Dynatech) and ELISA plate reader. The EC_{50} (concentration giving 50% of maximal observed

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RESULTS

For each compound tested, a full concentration–response curve was determined in the co-transfection assay, using the hGR cDNA and an MMTV-LUC reporter in CV-1 cells as described (Experimental). Representative data are shown in Fig. 1(A). In this assay, in the absence of added compound, the basal LUC activity is essentially undetectable (< 1 relative light unit, RLU). After exposure to fully efficacious glucocorticoids, e.g. dexamethasone, greater than 600 RLUs are obtained. If a control plasmid is substituted for that containing the hGR cDNA, fully efficacious concentrations of analogues give fewer than 5 RLUs (data not shown). Testing of the solvent at concentrations used to dissolve test substances had no effect on LUC activity (data not shown). When hGR is introduced, the concentration–response curve saturates, giving the maximal response above fully active concentrations. The transition from no measurable response to full response occurs over approximately two logs of concentration. Most active analogues give approximately full efficacy, although the partial efficacy displayed by fluocinolone [approx. 40%, Fig. 1(A)] was reproducible; fluocinolone produces no transactivation of MMTV-LUC in CV-1 in the absence of introduced hGR cDNA (data not shown). For each analogue, an EC_{50} was determined graphically as the concentration giving 50% of maximal effect for that compound. The EC_{50} data are compiled in Table 1; both the absolute EC_{50} (M) and a value normalized to that of hydrocortisone are given. Fluocinolone is the most potent compound (EC_{50} = 150 pM), being about 200-fold more potent than hydrocortisone. The least potent compound tested with detectable activity was progesterone (EC_{50} = 2.5 μM), almost 80-fold less potent than hydrocortisone.

Extracts of CV-1 cells infected with adenovirus engineered to encode the hGR cDNA were prepared as described (Experimental) and used to analyze the ability of the various steroids to displace 2.5 nM [^{3}H]dexamethasone. Representative data are shown in Fig. 1(B). Non-specific binding was < 2% of specific binding under these assay conditions [9]. The maximal binding was > 20-fold for extracts of cells infected with Ad/MLUC7 compared to mock-infected cell extracts [9]. For compounds capable of competing with [^{3}H]dexamethasone, including fluocinolone, > 95% of the specific

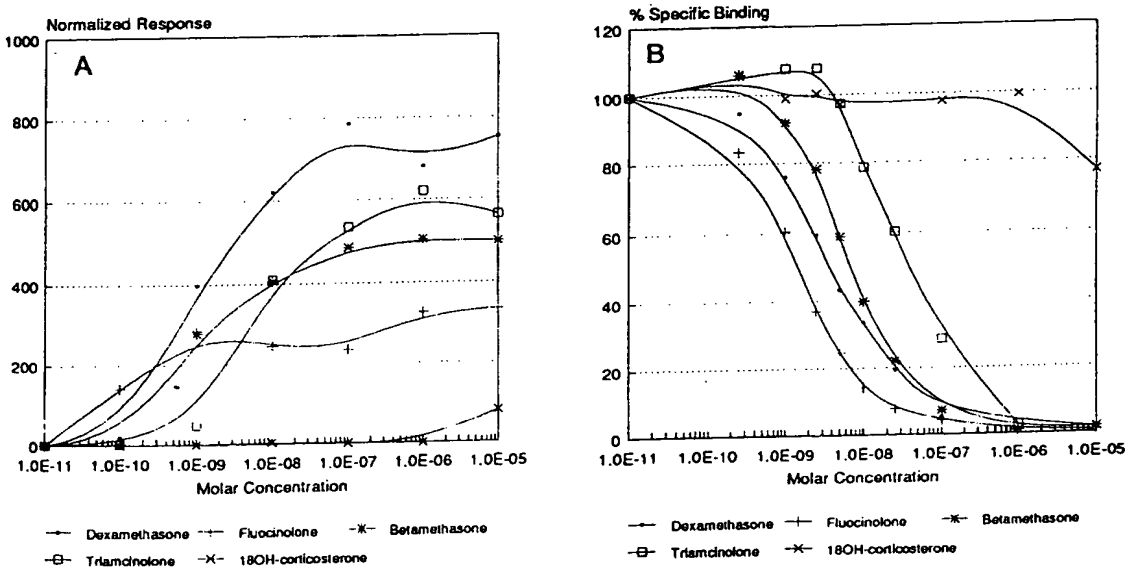

Fig. 1. Concentration dependence of transactivation and radioligand displacement by selected glucocorticoid analogues. Representative *in vitro* data are shown for several of the glucorticoid analogues tested. *Panel A*: dependence of hGR-mediated activation of the LUC reporter gene (ordinate) on concentration of glucocorticoid analogue in the culture medium (abscissa); and *Panel B*: concentration dependence of competitive displacement of 2.5 nM [^{3}H]dexamethasone from over-expressed hGR by several synthetic glucocorticoid analogues, expressed as percent of control binding (ordinate) as a function of added unlabeled competitor compound (abscissa).

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Table 1. Transactivation and binding of glucocorticoid analogues to hGR

Compound	Co-transfection EC_{50}	Co-transfection Normalized	Binding IC_{50}	Binding Normalized
Dexamethasone	1.2E-09	0.04	5.8E-09	0.12
Fluocinolone	1.5E-10	0.00	1.5E-09	0.03
Betamethasone	8.1E-10	0.03	7.0E-09	0.14
Triamcinolone	5.3E-09	0.17	4.0E-08	0.80
6α-Methyl prednisolone	1.3E-08	0.41	9.0E-09	0.18
Fludrocortisone	1.4E-08	0.44	1.3E-08	0.26
Prednisolone	2.7E-08	0.84	1.6E-08	0.32
Hydrocortisone	3.2E-08	1.00	5.0E-08	1.00
Corticosterone	4.7E-08	1.47	1.0E-07	2.00
Aldosterone	7.5E-08	2.34	7.2E-07	14.40
21-Deoxycortisol	2.5E-07	7.81	1.2E-07	2.40
11-Deoxycorticosterone	5.9E-07	18.44	5.0E-08	1.00
11-Deoxycortisol	7.6E-07	23.75	1.5E-07	3.00
Progesterone	2.5E-06	78.13	5.0E-08	1.00
Prednisone	> 1.0E-05		2.0E-06	40.00
Cortisone	> 1.0E-05		3.8E-06	76.00
Testosterone	> 1.0E-05		5.5E-06	110.00
Dihydrotestosterone	—		1.0E-05	200.00
Estriol	> 1.0E-05		> 1.0E-05	
Tetrahydrocortisone	> 1.0E-05		> 1.0E-05	
Tetrahydrocortisol	> 1.0E-05		> 1.0E-05	
Tetrahydrocorticosterone	> 1.0E-05		> 1.0E-05	
18-Hydroxydeoxycorticosterone	> 1.0E-05			
18-Hydroxycorticosterone	> 1.0E-05		> 1.0E-05	

Note: normalization to hydrocortisone.

binding was displaced over approximately a two log concentration range. Representative data are shown in Fig. 1(B). Table 1 gives graphically determined concentrations required to inhibit 50% of specific binding of 2.5 nM [^{3}H]dexamethasone (IC_{50}), both as absolute values (M) and normalized to the IC_{50} of hydroco tisone. IC_{50} values range over three logs, wi fluocinolone the most potent compound ar testosterone the least.

A comparison of normalized potency in tl co-transfection assay and in the competiti

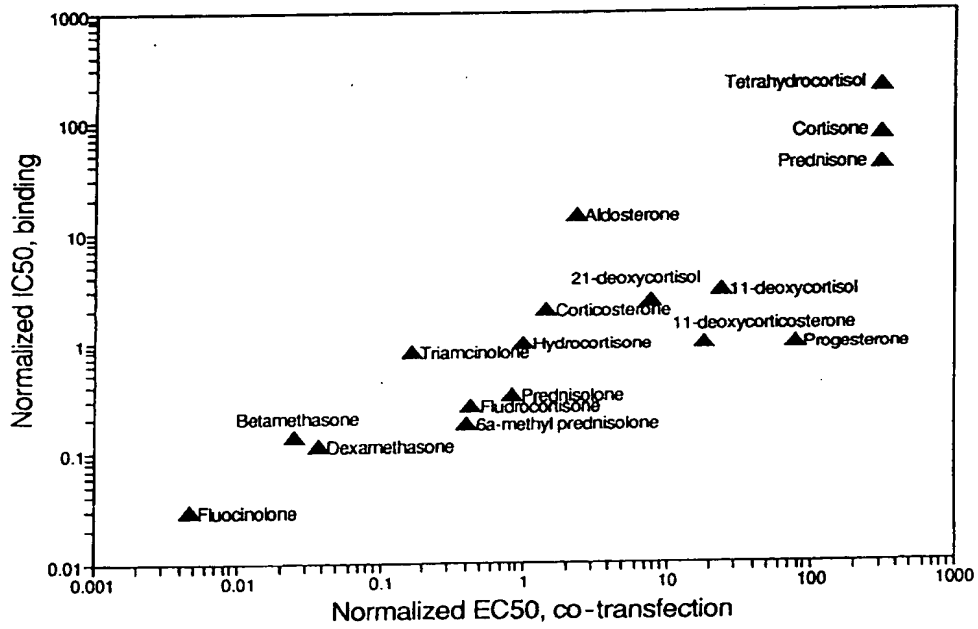

Fig. 2. Comparison of steroid binding and transactivation. Relative binding potency (ordinate) vs relative transactivation potency (abscissa). Competitive binding potency was determined for each analogue by measuring IC_{50}, i.e. the concentration required to inhibit by 50% specific binding of 2.5 nM [^{3}H]dexamethasone to extracts of CV-1 cells over-expressing hGR cDNA as described (Experimental). Relative binding potency was derived by normalizing these data to the IC_{50} of hydrocortisone. Transactivation potency was measured for each analogue in the co-transfection assay using hGR cDNA in CV-1 cells as described (Experimental). Data are expressed as EC_{50}, normalized to the EC_{50} of hydrocortisone.

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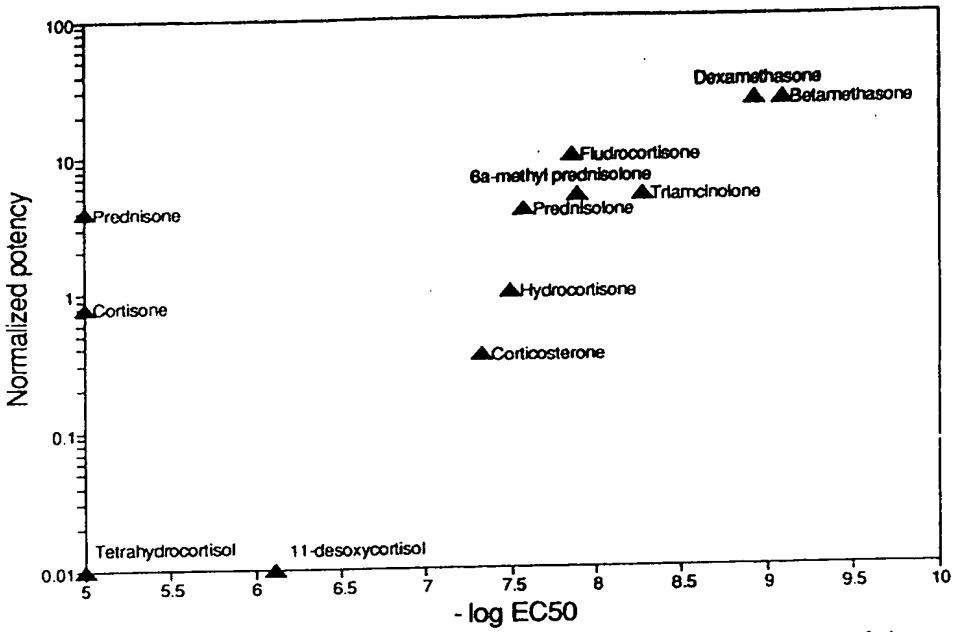

Fig. 3. Comparison of *in vivo* anti-inflammatory potency and *in vitro* hGR transactivation of glucocorticoid analogues. Relative anti-inflammatory potency *in vivo* (ordinate) vs *in vitro* transactivation potency (abscissa). Relative anti-inflammatory potency, normalized to that of hydrocarbons, is derived from reported values [11]. Transactivation potency was determined in the *in vitro* co-transfection assay using hGR cDNA introduced into CV-1 cells as described (Experimental). Values are expressed as—log EC_{50}.

binding assay for the various analogues (Fig. 2) reveals an excellent correlation between these two parameters. A notable exception to this correlation is progesterone, which is equipotent with hydrocortisone in displacing [^{3}H]dexamethasone, but about 80-fold weaker as a functional agonist of the hGR in the co-transfection assay.

Comparison of the biological potencies of the various steroids analyzed in the co-transfection assay *in vitro* compared to reported values of their *in vivo* anti-inflammatory potency ([11] based on dose in milligrams necessary for equal efficacy) is presented graphically in Fig. 3. Very good agreement is noted for all compounds, with the exception of prednisone and cortisone, which are much more potent *in vivo* than they are in the *in vitro* co-transfection assay in CV-1 cells.

DISCUSSION

The comparison of binding potency and transactivation agonist activity *in vitro* (Table 1 and Fig. 2) shows good correlation between the two for most of the 21 compounds examined. The partial agonist activity observed with fluocinolone (Fig. 1) does not appear to result from interaction of the compound with a subset of the introduced hGR, since fluocinolone gives no agonist activity in CV-1 cells in the absence of transfected hGR cDNA and is capable of displacing 100% of the specifically bound [^{3}H]dexamethasone from hGR-expressing CV-1 cell extracts. It is possible that the conformation of the fluocinolone–hGR complex is less effective than full agonists at interacting with other components of the transcriptional apparatus in CV-1 cells.

The present study underscores the feasibility of using the "*cis–trans*" assay for quantitative evaluation of potential hGR agonist. There are several significant theoretical advantages of the "*cis–trans*" assay over conventional radioligand binding assays. The most significant is that the assay determines not only whether a compound interacts with hGR but also the functional consequences of that interaction on gene expression, allowing the prediction of agonist and antagonist pharmacological effects. The assay, using hGR, can be expected to be less susceptible to potential species-related artifacts and inaccurate predictions than small-animal-based pharmacological studies. Finally, in screening for novel pharmacophores which might act as agonists or antagonists of the hGR, the "*cis–trans*" assay can detect any small molecules with functional consequences,

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whether or not they interact with the receptor in the natural hormone binding site. Competitive radioligand displacement assays can only detect such compounds if their binding results in allosteric effects on the ligand site.

The agreement between the relative potencies of the 21 analogues as activators of hGR-dependent transcription and as competitors with dexamethasone binding was remarkably close (Table 1 and Fig. 2). There were a few compounds which showed greater ability to displace dexamethasone binding than ability to transactivate MMTV-LUC in CV-1 cells. Progesterone was equipotent with hydrocortisone in binding, but 80-fold less potent in transactivation. These data suggest that progesterone binds to the hGR but doesn't lead to the allosteric changes in hGR conformation necessary for transactivation. This failure could be at the level of dissociation of hGR and heat shock protein or at the level of interaction with the GRE. The data suggest that progesterone might antagonize glucocorticoid activation of the hGR.

In the case of the compounds for which estimates of *in vivo* anti-inflammatory potency were available, there was remarkably good correlation with the *in vitro* results in the "*cis–trans*" assay (Fig. 3). This reflects the extent to which the model cell system mirrors systemic sites of *in vivo* action. Two notable exceptions to this were prednisone and cortisone, both of which had low activity in the CV-1 cell assay. Formally, this could reflect either catabolism to inactive derivatives by CV-1 cells or the absence of necessary metabolic activation *in vitro*. The inactive compounds both have carbonyl functions at position 11. In the "*cis–trans*" assay, their 11-hydroxylated analogues, prednisolone and corticosterone, were about two logs more potent. Prednisone and cortisone were also relatively ineffective in displacing [^{3}H]dexamethasone from hGR-containing extracts at 4°C *in vitro*, arguing against catabolism as the explanation of their lack of potency in the "*cis–trans*" assay. Prednisone and cortisone are known to require hepatic metabolic activation to prednisolone and corticosterone for *in vivo* activity [11]. This activating metabolic conversion apparently does not occur in CV-1 cells *in vitro*. With the exception of these compounds requiring metabolic activation, the "*cis–trans*" assay is surprisingly predictive of *in vivo* potency.

In the present study, close correlation was found in most cases between the relative potencies of the 21 compounds in functional agonist activity *in vitro* and in radioligand displacement. Furthermore, there was excellent correlation between *in vitro* data and reported *in vivo* anti-inflammatory data for these compounds. Introduction by infection or co-transfection of hGR cDNA and a suitable reporter into a receptor-deficient mammalian cell establishes a hormone-inducible transcription system which be utilized to quantitate the pharmacological efficacy and potency of potential ligands for the human glucocorticoid receptor.

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